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(71) Applicant: **INSTITUT BELKA AKADEMII NAUK SSSR**
Moskovskaya obl.
Puschino 142292(SU)

(72) Inventor: **BARANOV, Vladimir Ivanovich**
mikroraion D, 1-40
Moskovskaya obl.
Puschino, 142292(SU)
Inventor: **RYABOVA, Ljubov Anatolievna**
mikroraion G, 22-81
Moskovskaya obl.
Puschino, 142292(SU)
Inventor: **YARCHUK, Oleg Bronislavovich**
mikroraion G, 21-100
Moskovskaya obl.
Puschino, 142292(SU)
Inventor: **SPIRIN, Alexandr Sergeevich**
ul. Profsojuznaya, 43-1-10
Moscow, 117420(SU)

(74) Representative: **Goldin, Douglas Michael et al**
J.A. KEMP & CO.
14, South Square
Gray's Inn
London WC1R 5LX (GB)

(54) **METHOD FOR OBTAINING POLYPEPTIDES IN A CELL-FREE SYSTEM.**

(57) Polypeptides are obtained in a cell-free system containing exogenous RNA-polymerase, protein genes in the form of DNA molecules, nucleic acid and substrates. It contains, as substrates, ATP, GTP, CTP, UTP and amino acids. The products obtained in the system include the desired polypeptide AMP, GDP, CDP, UDP, pyrophosphate and non-organic phosphate. In the course of translation process, as substrates are consumed and products are obtained, products are removed from the system, containing the desired polypeptide, AMP, GDP, CDP, UDP, pyrophosphate and non-organic phosphate, while ATP, GTP, CTP, UTP and amino acids are simultaneously fed in the system, as substrates, to maintain their initial concentration.

EP 0 593 757 A1

Field of the Art

This invention relates molecular biology and bioengineering, and more particularly to methods of preparing polypeptides in cell-free translation system.

Said polypeptides are widely used in medicine as regulators of biological processes. Known in the prior art are, e.g., polypeptides activating the immune system, polypeptides which are neuromediators and transmitters, polypeptides regulating salt metabolism, etc. Polypeptides are also used in agriculture as biological stimulants, e.g., growth hormones. They are also used in bioelectronics, e.g., as rodopsin films.

State of the Art

Known in the art is a method for a preparative expression of cell genetic material by the method of genetic engineering based on invasion of a foreign DNA into a live cell, the genetic material of said foreign DNA being expressed by the apparatus of the host cell. This method is widely employed in commercial production of proteins.

However, the method has a limited application. This is associated with the complexity of isolation of the products of gene expression by the transformed cells, lethality of some specific products for the host cell, elimination of the transformed plasmids from the cell, proteolytic degradation or aggregation of the product of expression of the foreign gene.

It follows from the foregoing that the method of genetic engineering does not provide for possibilities of a preparative expression of any gene.

Known is another method of expression of genes based on the use of a cell-free system of continuous conjugated transcription/translation (Gene, 1989, v. 84, p. 463). This system is free of limitations imposed by a cell and ensures expression of substantially any gene in the form of a DNA molecule engineered in the required manner.

However, this method cannot be applied for cell-free eukaryotic systems. The matter is that upon expression of genes by the said method the use is made of endogenous RNA-polymerases of the object employed for preparation of the cell-free system of conjugated transcription/translation. This requires the use of special methods for isolation of the cell extract which ensure the maintenance of the activity of endogenous RNA-polymerases. Moreover, in eukaryotic cells the transcription and translation processes are, as a rule, dispersed in space and time: the transcription takes place in the cell core, while the translation occurs in the cell cytoplasm after relevant modifications of mRNA. Therefore up to the present all attempts to obtain a reliable system of conjugated transcription/translation based on eukaryotic cell extracts have been unsuccessful. The only method providing a reliable preparation of such extracts is based on the preparation of the S30 extract from bacterial cells of ***Escherichia coli***. Besides, the plasmid containing the gene coding for the specific product has a selection gene (the gene resistant to the action of any antibiotic) which is also under the promoter of the RNA-polymerase of *E. coli* and is expressed as efficiently as the gene coding for the specific product. As a result, in addition to the specific product, a side product is synthesized upon functioning of the system.

Known in the art is one more method of preparative synthesis of polypeptides based on the use of continuous cell-free translation system containing a template RNA as a nucleic acid. The method consists in preparing polypeptides in ribosome cell-free translation system containing ATP, GTP and amino acids as substrates accompanied by the formation of translation products in the system which include the specific product, AMP, GDP, pyrophosphate and inorganic phosphate. In the process of translation, translation products, including AMP, GDP, pyrophosphate, inorganic phosphate and the specific product, are removed from the system according to the consumption of substrates for the product formation with a simultaneous delivery in the system of substrates in the form of amino acids, ATP, GTP to maintain their initial concentration unchanged (Science, 1988, v. 242, p. 1162).

Said method makes it possible to carry out preparative synthesis of substantially any polypeptides in cell-free translation systems prepared from cells of any organisms.

However, the application of this method makes impossible the expression of the genetic material as DNA molecules. A template RNA is used in this method. This means that to realize the method, it is necessary to carry out an additional synthesis of template RNAs. As known, a template RNA is obtained from DNA molecules using transcription by RNA-polymerases. This is a labor-consuming and expensive process. Thus, at present the available methods do not permit to synthesize polypeptides using DNA molecules in any cell-free systems.

Disclosure of the Invention

The object of the invention is to develop such a method of preparation of polypeptides in cell-free systems which would ensure preparation of polypeptides with the use of DNA molecules in any cell-free system based on both prokaryotic and eukaryotic extracts.

This object is accomplished by provision of the method for preparation of polypeptides in the cell-free translation system containing a nucleic acid and ATP, GTP, and amino acids as substrates, with formation of translation products including the specific polypeptide, AMP, GDP, pyrophosphate and inorganic phosphate which are removed from the system according to the consumption of substrates with a simultaneous delivery of ATP, GDP and amino acids as substrates for maintenance of their initial concentration. According to the invention, the system also contains an exogenous RNA-polymerase as well as a nucleic acid in the form of protein genes as DNA molecules with promoter sites specific to the above polymerase, CTP and UDP as substrates and, in addition, CDP and UDP as products.

Prokaryotic and eukaryotic cell-free translation systems are used as cell-free translation systems according to the invention. E.g., systems based on *E. coli* extracts can be used as prokaryotic cell-free systems, and systems based on extracts from wheat embryos or on lysates from *E. coli* rabbit reticulocytes can be used as eukaryotic cell-free systems. The ratio of the components in the reaction mixture, ion and temperature conditions of the synthesis are optimal for the organisms from which cell-free systems and exogenous RNA-polymerases are prepared. The range of these conditions is rather wide.

The method implies the use of an exogenous phage RNA-polymerase, e.g., phage T7 RNA-polymerase or phage SP6 RNA-polymerase, as an exogenous RNA-polymerase.

As said, in some cases it is expedient to use a prokaryotic cell-free system based on *E. coli* extracts. Such a translation system contains an endogenous RNA-polymerase. To prevent the formation of additional translation products, an additional delivery of an inhibitor of the endogenous RNA-polymerase should be used. E.g., rifampicin is used as an inhibitor of the prokaryotic endogenous RNA-polymerase.

The nucleic acid employed in the system in represented by the protein genes in the form of DNA molecules with promoter sites specific to an exogenous RNA-polymerase. Such protein genes can be, e.g., DNA molecules obtained by amplification of a DNA fragment and a plasmid DNA can also be used.

The proposed method has no disadvantages of the genetic engineering method and known methods of preparative synthesis of polypeptides in continuous cell-free translation systems. It provides preparation of polypeptides within various cell-free systems without a preliminary synthesis of template RNA molecules. Due to the choice of the components, the synthesis of a template RNA proceeds directly in the cell-free system.

The proposed method ensures the preparative synthesis of polypeptides at a constant rate during tens of hours with an yield of the functionally active product (polypeptide) of 1 to 10 nmol per 1 ml of the reaction mixture and can be employed in commercial production of preparing polypeptides in any cell-free systems.

Description of the Drawings

The invention will further be described with reference to the appended drawings in which:

Figs. 1, 2, 4, 5 and 7 represent graphic dependencies of the quantity of the synthesized polypeptide in nanomoles on the time of the synthesis in hours.

Fig. 3 is a photograph of the fluorogram of SDS-urea-polyacryl aminde gel illustrating the distribution of the translation products according to their molecular weight.

Fig. 6 is a photograph of a thin-layer chromatography radioautograph illustrating the distribution of the products of the reaction catalyzed by chloramphenicol acetyl transferase enzyme (Anal. Biochem., 1987, v. 160, p. 65-67).

Preferable Variant of Embodiment of the Invention

The technological aspect of the method of preparing polypeptides in cell-free translation systems is simple and the method can be realized as follows.

Extracts of prokaryotic and eukaryotic cells containing ribosomes and all components of the translation machinery but free of endogenous mRNA and DNA are prepared using the known methods. Low molecular weight components including amino acids, ATP, GTP, CTP, UTP, exogenous RNA-polymerase, the gene in the form of a DNA molecule with a promoter site specific to the above polymerase are added to the extract.

The cell-free system is protected from the environment by a porous barrier with pores sufficient to pass specific products. The reaction vessel for the synthesis of polypeptides can be a reservoir in which the cell-free system protected by a porous barrier is placed. A porous barrier can be made either of organic or inorganic material. E.g., ultrafiltration membranes, hollow fibers, microcapsules or films whose shell represents polyelectrolyte complexes can be used as porous barriers. The cell content is then heated to the required temperature.

During the synthesis, the translation products are removed from the reaction vessel through the porous barrier. Simultaneously, substrates from a separate reservoir are supplied into the system to

maintain their initial concentration. The specific product withdrawn from the system is concentrated and purified.

The method is illustrated by the following examples:

Example 1.

1 ml of the reaction mixture contains 350 µl of the S30 extract from *E. coli*, 0.1 mg of tRNA, 0.04 mg of the DNA fragment containing the gene of the β-lactomase precursor and the promoter for T7 polymerase obtained according to the technique (Molecular Cloning, 1989, Cold Spring Harbor Laboratory Press, ed. J. Sambrook, E.F. Fritsh, T. Maniatis, p. 1-21), 30,000 U of T7 polymerase, 0.1 mg of pyruvate kinase 50 U of ribonuclease inhibitor from human placenta, 5 µg of each of the protease inhibitors (leupeptin, chymostatin) and α2-macroglobulin in buffer A: 50 mM Tris Ac, pH 7.5, 14 mM $MgCl_2$, 100 mM KAc, 2 mM $CaAc_2$, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 10 mM phosphoenolpyruvate, 4 mM dithiothreitol, 50 µM spermidine, 10 µg leukovorin, 40 µM rifampicin, 30 µM [^{3}H]Leu with specific activity 1.7 Cu/mmol and 30 µM each of the other 19 amino acids.

0.5 ml of the cell-free system is placed in a cell for ultra-filtration and the peptide is synthesized at 37°C. The translation products, including the specific product and the products of decomposition, are withdrawn through a porous barrier with a simultaneous delivery of substrates in the form of ATP, GTP, CTP, UTP and amino acids in buffer A during 20 hours. As a result, protein of β-lactomase is obtained. The substrates are supplied at a rate of 2 ml/h.

During the entire synthesis, the specific product is synthesized at a constant rate. The dependence of the amount of the obtained product on the time of synthesis is given in Fig. 1. The abscissa axis shows the time in hours and the ordinate axis shows the amount of the obtained product in nanomoles. As a result, 250 pmol of β-lactomase are synthesized during 20 hours of the system operation.

Example 2.

1 ml of the reaction mixture contains 350 µl of the S30 extract from *E. coli*, 0.2 mg of tRNA, 0.1 mg of plasmid containing the gene of dihydrofolate under the promoter of SP6 polymerase obtained according to the technique described (Molecular Cloning, 1989, Cold Spring Harbor laboratory Press, ed. J. Sambrook, E.F. Fritsh, T. Maniatis, p. 1-21), 20,000 U of SP6 polymerase, 0.1 mg of pyruvate kinase, 50 U of ribonuclease inhibitor from human placenta, 5 µl of each protease inhibitors (leupeptin, chymotrypsin) and α2-macroglobulin in buffer A: 50 mM Tris-Ac, pH 7.5, 14 mM $MgCl_2$, 100 mM KAc, 2 mM $CaAc_2$, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 10 mM phosphoenolpyruvate, 4.0 mM dithiothreitol, 50 µM spermidine, 10 µg leukovorin, 40 µM rifampicin, 30 µM [^{35}S]Met with specific radioactivity of 800 mCu/mmol and 30 µM of each of the other 19 amino acids.

0.5 ml of the cell-free system is placed in a cell for ultrafiltration and the polypeptide is synthesized at 37°C. The translation products, including the specific product and the products of decomposition, are removed from the system through a porous barrier with a simultaneous delivery of substrates such as ATP, GTP, CTP, UTP and amino acids in buffer A into the reaction mixture during 20 hours. As a result, protein of dihydrofolate reductase is obtained. The substrates are delivered at a rate of 1.5 ml/h.

During the entire synthesis, the specific product is synthesized at a constant rate. The dependence of the amount of the product obtained on the time of the synthesis is given in Fig. 2. The abscissa axis shows the time of the synthesis in hours and the ordinate axis shows the amount of the synthesized product in nanomoles.

As a result, 680 pmol of dihydrofolate reductase are synthesized during 24 hours. The synthesized enzyme is active functionally. Its specific activity was measured as described (Nature, 1960, v. 188, p. 231-232) and was $0.13 \cdot 10^{-4}$ activity units per picamole of the enzyme synthesized.

In this case, the plasmid utilized contains the gene of dihydrofolate reductase under the promoter of SP6 polymerase and the gene of β-lactomose under the promoter of *E. coli* RNA-polymerase. Since rifampicin, an inhibitor of *E. coli* RNA-polymerase, is present in the system, no synthesis f β-lactomase takes place. Therefore, only the specific product is synthesized in the system.

The results of the electrophoretic analysis of the polypeptide obtained in 2, 4, 6, 8 and 10 hours after the beginning of the system operation are represented in Fig. 3.

Example 3.

1 ml of the incubation mixture contains 320 µl of wheat embryo extracts, 0.1 mg of the plasmid with the gene of dihydrofolate reductase under the promoter of SP6 polymerase obtained by the method described (Molecular Cloning, 1989, Cold Spring Harbor Laboratory Press, ed. J. Sambrook, E.F. Fritsh, T. Maniatis, p. 1-21), 20,000 U of SP6 polymerase, 0.1 mg of pyruvate kinase, 50 U of ribonuclease inhibitor from human placenta, 5 µg

of each of the protease inhibitors (leupeptin, chymotrypsin) and α2-macroglobulin in buffer A: 40 mM HEPES, pH 7.6, 2.5 mM $MgAc_2$, 70 mM KAc, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 0.25 mM spermidine, 4.0 mM dithiothreitol, 6 mM creatin phosphate, 20 µM [^{14}C]Leu with specific radioactivity of 21 µCu/mmol, 20 µM of each of the other 19 amino acids.

0.5 ml of the cell-free system is placed in a cell for ultrafiltration and the polypeptide is synthesized at 24 °C. The translation products, including the specific product and the products of decomposition, are removed from the system through a porous barrier with a simultaneous delivery of substrates such as ATP, GT, CTP, UTP and amino acids in buffer A into the reaction mixture during 24 hours. As a result, protein of dihydrofolate reductase is obtained. The substrates are delivered at a rate of 2.0 ml/h.

During the entire synthesis the product is synthesized at a constant rate. The dependence of the amount of the product obtained on the time of the synthesis is given in Fig. 4. THe abscissa axis shows the time of the synthesis in hours and the ordinate axis shows the amount of the product obtained in nanomoles. As a result, 5 nmol of dihydrofolate reductase were synthesized. The synthesized enzyme was active functionally. The specific activity of the enzyme obtained was measured as described (Nature, 1960, v. 188, p. 231-232). It was $0.25 \cdot 10^{-4}$ activity units per picamole of the enzyme synthesized.

Example 4.

1 ml of the incubation mixture contains 600 µl of lysate from rabbit reticulocytes, 0.1 ml of plasmid with the gene of chloramphenicol acetyl transferase under the promoter of SP6 polymerase obtained according to the method described (Molecular Cloning, 1989, Cold Spring Harbor Laboratory Press, ed. J. Sambrook, E.F. Fritsh, T. Maniatis, p. 1-21), 30,000 U of SP6 polymerase, 0.1 mg of pyruvate kinase, 50 U of the ribonuclease inhibitor from human placenta, 5 µg of each of the protease inhibitors (leupeptin, chymotrypsin) and α2-macroglobulin in buffer A: 25 mM HEPES, pH 7.6, 1.5 mM $MgAc_2$, 100 mM KAc, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 0.25 mM spermidine, 4.0 mM dithiothreitol, 6 mM creatin phosphate, 20 µM [^{35}S]Met with specific radioactivity of 800 mCu/mmol, 20 µM of each of the other 19 amino acids.

0.5 ml of the cell-free system is placed in a cell for ultrafiltration and the polypeptide is synthesized at 37 °C. The translation products, including the specified product and the products of decomposition, are removed through a porous barrier with a simultaneous delivery of substrates such as ATP, GTP, CTP, UTP and amino acids in buffer A into the reaction mixture during 34 hours. As a results, a protein of chloramphenicol acetyl transferase is obtained. The substrates are delivered at a rate of 1.5 ml/h.

During the entire synthesis the specific product is synthesized at a constant rate. The dependence of the amount of the product obtained on the time of the synthesis is given in Fig. 5. The abscissa axis shows the time of synthesis in hours and the ordinate axis shows the amount of the product obtained in nanomoles. As a result, 2.5 nmol of chloramphenicol acetyl transferase was synthesized. The enzyme synthesized was active functionally. The functional activity of the enzyme obtained was measured as described (Anal. Biochem., 1987, v. 160, p. 65-67). The results of the analysis of functional activity of the enzyme obtained in 0, 0.5, 5, 7, 9 and 12 hours performed using thin-layer chromatography with a following radioautography are represented in Fig. 6.

Example 5.

1 ml of the incubation mixture contains 600 µl of lysate from rabbit reticulocytes, 0.1 mg of plasmid with the gene of dihydrofolate reductase under the promoter of SP6 plasmid obtained according to the method described (Molecular Cloning, 1989, Cold Spring Harbor Laboratory Press, ed. J. Sambrook, E.F. Fritsh, T. Maniatis, p. 1-21), 30,000 U of SP6 polymerase, 0.1 mg of pyruvate kinase, 50 U of the ribonuclease inhibitor from human placenta, 5 µg of each of the protease inhibitors (leupeptin, chymotrypsin) and α2-macroglobulin in buffer A: 25 mM HEPES, pH 7.6, 1.5 mM $MgAc_2$, 1 mM ATP, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 0.25 mM spermidine, 4.0 mM dithiothreitol, 6 mM creatin phosphate, 20 µM [^{14}C]Leu with specific radioactivity of 21 mCu/mmol, 20 µM each of the other 19 amino acids.

0.5 ml of the cell-free system is placed in a cell for ultrafiltration and the polypeptide is synthesized at 37 °C. The translation products, including the specific product and the products of decomposition are removed through a porous barrier with a simultaneous delivery of substrates such as ATP, GTP, CTP, UTP and amino acids in buffer A during 20 hours. As a result, a protein of dihydrofolate reductase is obtained. The substrates are delivered with a rate of 2.0 ml/h.

During the entire synthesis the product is synthesized at a constant rate. The dependence of the amount of the product obtained on the time of the synthesis is given in Fig. 7. The abscissa axis shows the time of the synthesis in hours and the ordinate axis shows the amount of the product

obtained in nonomoles. As a result, 7.0 nmol of dihydrofolate reductase is synthesized. The enzyme synthesized is active functionally. The specific activity of the enzyme obtained measured as described (Nature, 1960, v. 188, p. 231-232) is $0.3 \cdot 10^{-4}$ activity units per picamole of the enzyme synthesized.

Industrial Applicability

The polypeptide obtained accordingly to the invention can be used in medicine, agriculture, and bioelectronics. The method is a multi-purpose one as it ensures synthesis of a template RNA in the process of polypeptide preparation.

Claims

1. Method of preparing polypeptides in cell-free translation system containing nucleic acid and substrates such as ATP, GTP and amino acids accompanied by synthesis of products including a specific polypeptide, AMP, GDP, pyrophosphate and inorganic phosphate removed from the system with consumption of substrates with a simultaneous delivery into the system of substrates such as ATP, GTP and amino acids to maintain their initial concentration characterized by the presence in the system of exogenous RNA-polymerase and protein genes in the form of DNA molecules with promoter sites specific to the above polymerase as a nucleic acid, CTP and UTP as substrates and additional CDP and UDP as products. The system is protected by a porous barrier.

2. The method of claim 1 in which the system contains an exogenous phage RNA-polymerase as an exogenous RNA-polymerase.

3. The method of claim 2 in which the system contains an exogenous phage SP6 RNA-polymerase as an exogenous phage RNA-polymerase.

4. The method of claim 2 in which the system contains an exogenous phage T7 RNA-polymerase as an exogenous RNA-polymerase.

5. The method of claim 1 in which the system contains DNA molecules obtained by amplification of the DNA fragment as the mentioned protein genes represented by DNA molecules.

6. The method of claim 1 in which the system contains a plasmid DNA as the mentioned protein genes represented by DNA molecules.

7. The method of claim 1 in which prokaryotic cell-free system is used.

8. The method of claim 7 in which cell-free system based on *E. coli* extracts is used as prokaryotic cell-free system.

9. The method of claim 8 in which the system contains additional inhibitors of endogenous RNA-polymerases.

10. The method of claim 1 in which eukaryotic cell-free system is used.

11. The method of claim 10 in which cell-free system based on plant cell extracts is used as eukaryotic cell-free system.

12. The method of claim 11 in which extracts from wheat embryos are used as plant cells.

13. The method of claim 10 in which cell-free system base don animal cell lysates is used as eukaryotic cell-free system.

14. The method of claim 13 in which rabbit reticulocyte lysates are used as animal cell lysates.

15. The method of claim 1 in which the porous barrier is made of inorganic material.

16. The method of claim 1 in which the porous barrier is made of organic material.

17. The method of claim 1 in which the porous barrier is made of a combination of organic and inorganic material.

18. The method of claim 1 in which the porous barrier represents planar membranes.

19. The method of claim 1 in which the porous barrier represents hollow fibers.

20. The method of claim 1 in which the porous barrier represents porous granules.

21. The method of claim 1 in which the porous barrier represents capsules.

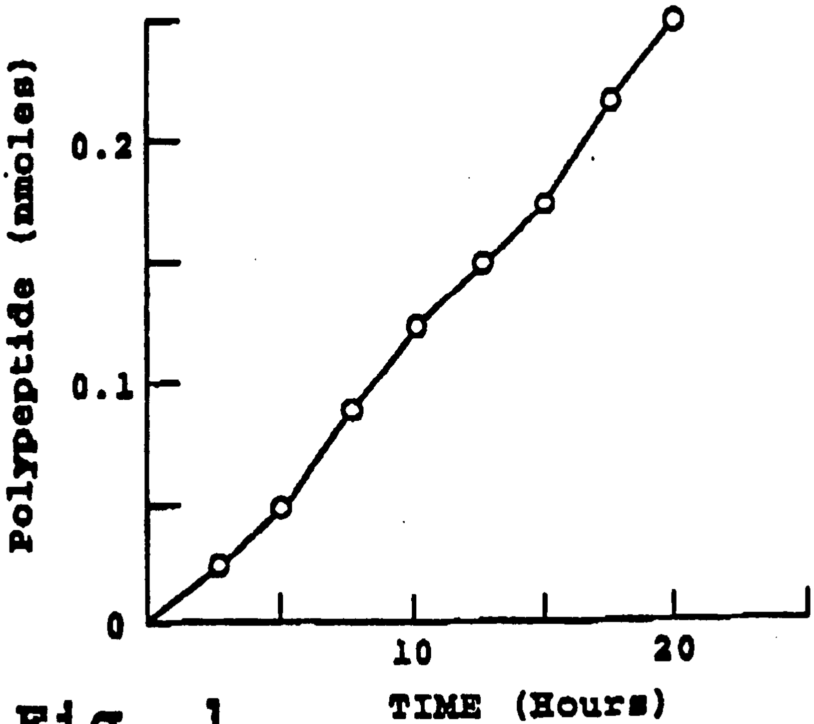

Polypeptide (nmoles)
0.2
0.1
0
10
20
TIME (Hours)

Fig. 1

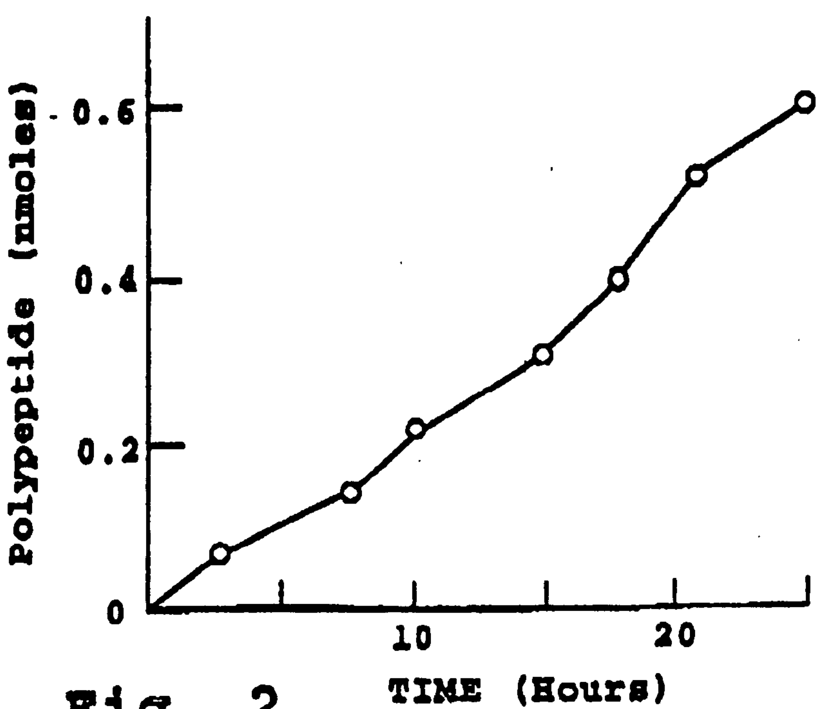
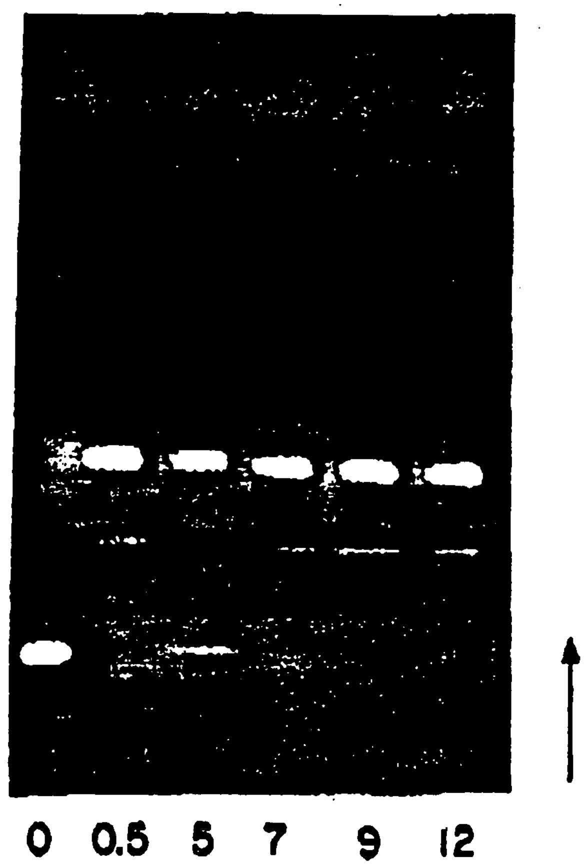

Polypeptide (nmoles)
0.6
0.4
0.2
0
10
20
TIME (Hours)

Fig. 2

Fig. 3

Fig. 6



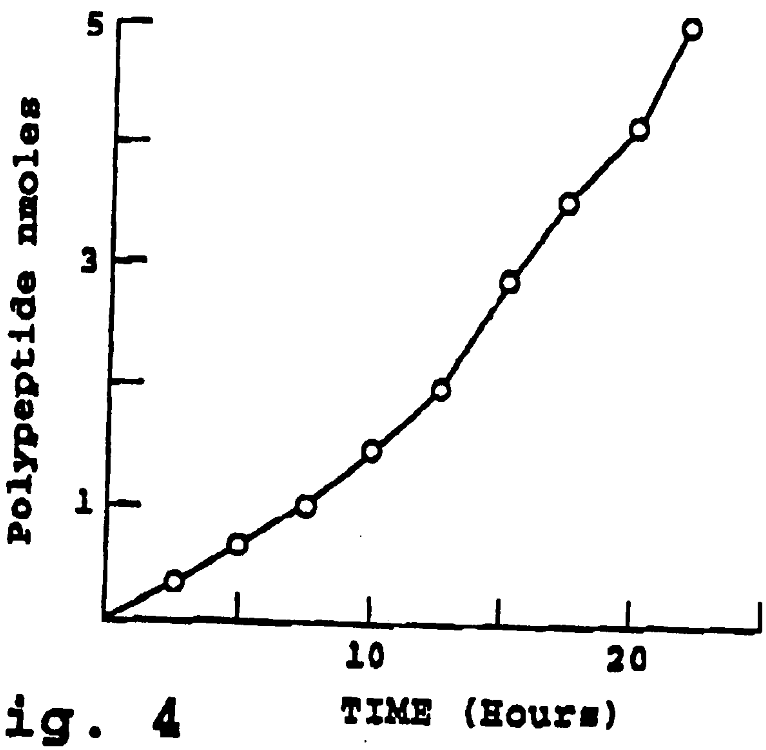

5
3
1
Polypeptide nmoles
10
20
TIME (Hours)

Fig. 4

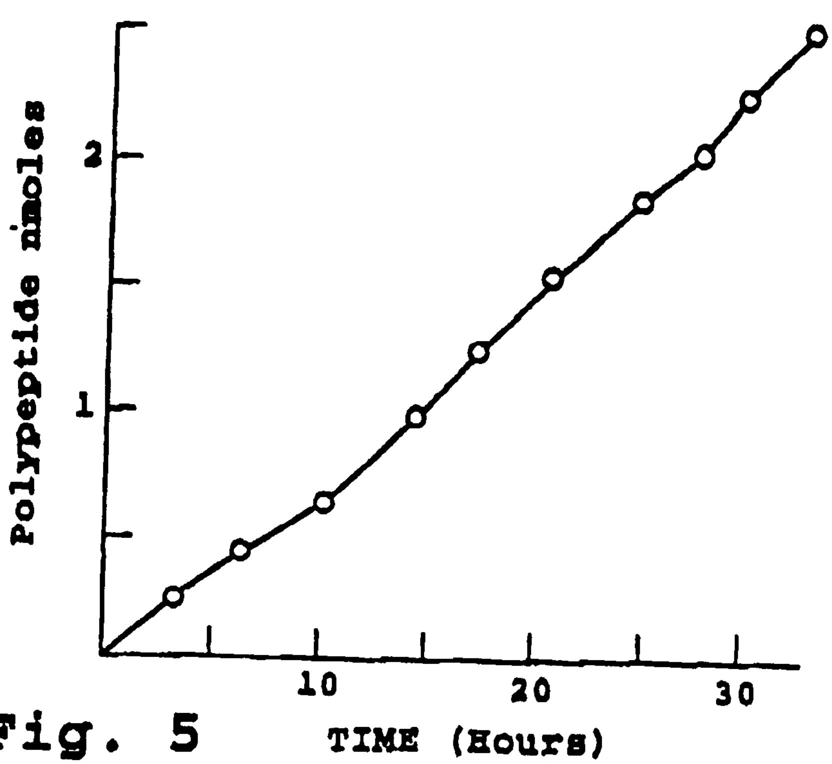

2
1
Polypeptide nmoles
10
20
30
TIME (Hours)

Fig. 5

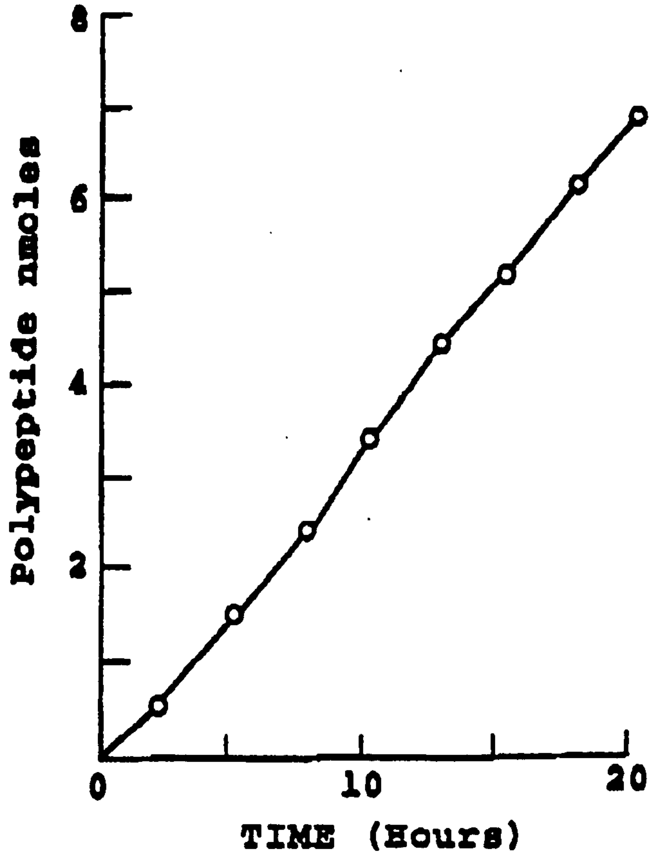


Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/SU 90/00151

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5 C12P 21/00; C12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC5	C12P 21/00, 21/02; C12 N 15/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	"Biopolimery i kletka", Vol. 4, No.3, 1988, Naukova dumka (kiev). A.P.Potapov et al. "Sravnitelnoe izuchenie matrichnoi aktivnosti poli (u) i poli (dT) v beskletochnykh beloksintezirujuschikh sistemakh iz Escherichia coli i zarodyshei pshenitsy", pages 133-138	1-20
A	"Nucleic Acids Research", V.9, N18, 1981 IRL Press Limited (London), Julie M.Pratt et al. "Identification of gene products programmed by restriction endonuclease DNA fragments using an E.coli in vitro system", page 4459	1-20
A	"Transkriptsia i translyatsia. Metody" (Pod. red, B.Keimsa, S.Sigginsa, 1987, Mir, (Moscow) pages 216-217	1-20

./..

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 September 1990 (27.09.90)	12 November 1990 (12.11.90)
International Searching Authority ISA/SU	Signature of Authorized Officer

International Application No. PCT/SU 90/00151

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	"SCIENCE", V.242,25 November, 1988 A.S.Spirin et al. "A Continuous Cell-Free Translation System Capable of Producing Polypeptides in High Yield", pages 1162-1164	1-20
A	WO,A1,88/08453 (Institut Belka AN SSSR), 3 November 1988 (03.11.88), the abstract	1-20

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers ...., because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers ...., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.